Are Cytotoxicity and Interferon Inducing Activity of Poly(I).Poly(C) Invariably Linked in Interferon-treated L cells?*

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SUMMARY

Interferon-treated L cells exhibit a specific enhanced susceptibility to the cytotoxic and interferon inducing activities of double-stranded RNAs such as poly(I).poly(C). These activities remained closely linked through widely varying assay conditions, involving, for example, different time and dosage schedules of poly(I).poly(C), suggesting that there is at least one common step in the mechanisms leading to interferon formation and toxicity in interferon-primed cells exposed to poly(I).poly(C). However, some procedures such as addition of metabolic inhibitors (actinomycin D, cycloheximide) and repeated administration of poly(I).poly(C) suppressed the interferon inducing capacity of poly(I).poly(C) without a concomitant decrease of toxicity. Other procedures such as brief treatment of the cells with interferon or DEAE-dextran permitted full expression of the interferon inducing activity of poly(I).poly(C) without any sign of toxicity. The latter results suggest that the mechanisms underlying interferon production and toxicity of poly(I).poly(C) in interferon-treated L cells diverge from a certain point onward.

INTRODUCTION

Mouse L cells are not fully responsive to the interferon inducing activity of double-stranded (ds) RNAs such as poly(I).poly(C) unless they have been treated (primed) with interferon (Rosztoczy & Mcs, 1970; Rosztoczy, 1971; Stewart, Gosser & Lockart, 1971a,b, 1972a; Margolis, Oie & Levy, 1972; De Clercq, Stewart & De Somer, 1973). However, interferon treatment renders L cells also more sensitive to the toxic effects of dsRNAs (Stewart, De Clercq & De Somer, 1973a,b; Stewart et al. 1972b, 1973c). Therefore, the question arises whether the cytotoxic and interferon inducing activities of poly(I).poly(C) and other dsRNAs in interferon-primed L cells are necessarily correlated or can be uncoupled by appropriate changes in the assay conditions.

Studies have now been undertaken to explore the possibility that the toxicity and interferon inducing capacity of poly(I).poly(C) in interferon-primed L cells are invariably linked. These studies should contribute to a better insight into one of the most fundamental problems of interferon research: is the production of interferon a specialized function of the normal healthy cell or should it merely be regarded as the consequence of a toxic alteration of the cell? The experiments to be described herein are also pertinent to the questions whether the therapeutic ratio (toxicity/antiviral activity ratio) of poly(I).poly(C) can be

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improved and whether dsRNAs could be developed with a higher therapeutic ratio than that of poly(I).poly(C).

METHODS

Materials and preparation. The source of the cells (mouse L 929), virus (VSV: vesicular stomatitis virus) and compounds [poly(I), poly(C), poly(I).poly(C), cycloheximide, actinomycin D, DEAE-dextran] has been documented previously (Stewart et al. 1973a). Mouse interferon was prepared in L 929 cell cultures inoculated with NDV (Newcastle disease virus, Kumarov strain) according to established procedures (Stewart et al. 1971b).

Assay of interferon inducing activity and toxicity. The general procedure for assaying the interferon inducing activity and toxicity of poly(I).poly(C) was the following: mouse L 929 cells, grown to confluency in 60 mm plastic Petri dishes (in MEM (minimal essential medium) supplemented with 10% calf serum) were treated for 16 h with 200 reference research units/ml of mouse interferon (in MEM + 3% calf serum, 2 ml/Petri dish), washed, exposed to 10 μg/ml of poly(I).poly(C) (in MEM, 1 ml/Petri dish) for 1 h, washed again, and incubated with MEM + 3% calf serum (4 ml/Petri dish) for 20 to 24 h. At this time cytotoxicity was recorded and the supernatant fluids were harvested for determination of the interferon titres.

Cytotoxicity was recorded semi-quantitatively as amount of cell destruction: -, none; +, 0 to 25% (+ if close to 0%); ++, 25 to 75% (+ + if close to 25%, + + + if close to 75%); ++++, 75 to 100% (+ + + + if close to 100%); +++++, 100% destruction of cell monolayers.

Interferon activity was determined by VSV plaque reduction in L 929 cells, 50% plaque reduction corresponding to 1 interferon unit. Interferon activity is presented as percent of maximum interferon response obtained in each particular assay. The presence of residual poly(I).poly(C) in the interferon samples was eliminated by pancreatic ribonuclease A treatment of the samples (no loss of antiviral activity after exposure of the samples to 40 μg/ml of pancreatic RNase for 1 h at 37°C in 4 × 10⁻⁶ M-EDTA) and titration of the samples in heterologous (PRK, primary rabbit kidney) cells (almost complete loss of antiviral activity). The antiviral effect detected with the mouse interferon samples in PRK cells was equivalent to the direct antiviral effect of approx. 0.02 μg/ml of poly(I).poly(C) in these cells. This amount represents 1/500 of the input poly(I).poly(C) and cannot account for the antiviral activity of the mouse interferon samples, since poly(I).poly(C) itself did not exhibit a direct antiviral effect in L 929 cells at a concentration lower than 1 μg/ml.

RESULTS

The responsiveness of interferon-primed L 929 cell cultures to the cytotoxic and interferon-inducing properties of poly(I).poly(C) has been measured in a number of widely varying assay conditions, differing from the general procedure outlined in Methods.

Interferon production and toxicity were generally determined at 20 to 24 h after the initial 1 h contact period of poly(I).poly(C) with the cells. When measurements were made at shorter times after this initial contact period, it appeared that interferon production and toxicity gradually increased up to 5 to 7 h, at which time both activities reached their peak values (Fig. 1).

Reduction of the contact period of poly(I).poly(C) with the cells from 60 min to 30, 10, 3 or 1 min led to a gradual decrease of both interferon production and toxicity (Fig. 2a).
Poly(I).poly(C): interferon and toxicity

Reduction of the dosage at which poly(I).poly(C) was applied to the cells was also paralleled by a gradual decrease in interferon production and cytotoxicity (Fig. 2b).

In accordance with previous findings (Stewart et al. 1971a), a 2 h incubation period with interferon (200 units/ml) sufficed to fully prime L 929 cells for the interferon inducing activity of poly(I).poly(C) (Fig. 3a). However, considerably longer exposure times (16 h) were required to fully prime the cells for the cytotoxic effects of poly(I).poly(C). No marked differences were noted in the dose dependence of the priming activities of interferon on either interferon induction or toxicity (Fig. 3b). That the antiviral and priming activities of interferon are equally dose-dependent, has also been demonstrated in earlier studies (Stewart et al. 1973c).

The antiviral activity of poly(I).poly(C) in cultured cells can be restored or even increased if the constituent homopolymers are administered separately (De Clercq & De Somer, 1971). It has also been shown that, upon sequential administration, poly(I) and poly(C) do not act independently, but reunite at the cellular level, most probably at the outer cell membrane (De Clercq & De Somer, 1972a). Sequential administration of poly(I) and poly(C) to interferon-primed L 929 cells resulted in an interferon response comparable to that obtained with the poly(I).poly(C) complex itself, provided that the time interval between the administration of the complementary homopolymers was limited to less than 2 h (Fig. 4). The interferon response gradually decreased with longer time intervals and disappeared completely if the second polymer was added 16 h after the first. The degree of cytotoxicity closely matched the interferon production pattern.

Exposure of the cells to poly(I).poly(C) prior to interferon treatment abolished the priming effect of interferon on the subsequent interferon response to a second pulse of
Fig. 2. Interferon induction and cytotoxicity of poly(I).poly(C) in interferon-primed L 929 cells. 
(a) Poly(I).poly(C) incubated on the cells for varying times (as indicated in the abscissa). (b) Poly(I).
poly(C) applied at different doses (as indicated in the abscissa) for 16h. 

poly(I).poly(C) (Table 1). Interestingly, the priming effect of interferon on the cytotoxicity 
of poly(I).poly(C) was not reversed by the preceding exposure of the cells to poly(I).poly(C).

Actinomycin D added to the cells together with poly(I).poly(C) prevented the interferon 
inducing activity of poly(I).poly(C) but failed to reduce its cytotoxicity (Table 2), indicating 
that RNA synthesis is required for interferon production but not for toxicity (see also
Fig. 3. Interferon induction and cytotoxicity of poly(I), poly(C) in interferon primed L 929 cells. (a) Cells exposed to interferon (200 U/ml) for various times (as indicated in the abscissa). (b) Cells exposed to different concentrations of interferon (as indicated in the abscissa).

Stewart et al. 1973a). Similarly, cycloheximide present during the whole interferon production period suppressed interferon formation but did not affect the cytotoxicity of poly(I), poly(C) (Table 2), pointing to the need of protein synthesis for interferon production but not for toxicity (see also Stewart et al. 1973a). Neither cycloheximide alone nor actinomycin D alone caused a toxic alteration of the cells.

DEAE-dextran added to the cells immediately after their treatment with interferon and before their contact with poly(I), poly(C) reduced the interferon inducing activity of
poly(I)·poly(C) and, concomitantly, protected the cells against the toxic influence of the polynucleotide (Table 3). However, interferon-primed cells which were exposed to a relatively high dose of DEAE-dextran, were markedly more responsive to interferon induction than to the cytotoxicity of poly(I)·poly(C), and cells, which had not been primed with interferon before they were exposed to DEAE-dextran, responded only to the interferon inducing capacity of poly(I)·poly(C) without concomitant toxicity (Table 3).
Table 1. *Effect of repeated administration* on interferon inducing activity and toxicity of poly(I).poly(C) in interferon-primed L929 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Interferon inducing activity (%)</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>First dose of poly(I).poly(C) (µg/ml)</td>
<td>Interferon priming</td>
<td>Second dose of poly(I).poly(C) (µg/ml)</td>
</tr>
<tr>
<td>100</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

* Poly(I).poly(C) was applied twice, each time for 1 h, with 20 h interval. The cells were incubated with interferon (20 U/ml) during this interval period.

Table 2. *Effect of metabolic inhibitors* on interferon inducing activity and toxicity of poly(I).poly(C) in interferon-primed L929 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Interferon inducing activity (%)</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon induction period</td>
<td>Interferon production period</td>
<td></td>
</tr>
<tr>
<td>Poly(I).poly(C)</td>
<td>---</td>
<td>100</td>
</tr>
<tr>
<td>Poly(I).poly(C)</td>
<td>---</td>
<td>3</td>
</tr>
<tr>
<td>Poly(I).poly(C) + actinomycin D</td>
<td>---</td>
<td>Actinomycin D</td>
</tr>
<tr>
<td>Poly(I).poly(C) + cycloheximide</td>
<td>---</td>
<td>Cycloheximide</td>
</tr>
</tbody>
</table>

* Actinomycin D at 5 µg/ml, cycloheximide at 20 µg/ml.

Table 3. *Effect of DEAE-dextran* on interferon inducing activity and toxicity of poly(I).poly(C) in interferon-primed and unprimed L929 cells

<table>
<thead>
<tr>
<th>DEAE-dextran concentration (µg/ml)</th>
<th>Interferon inducing activity (%)</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon-primed cell cultures</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>+ +</td>
</tr>
<tr>
<td>50</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>250</td>
<td>50</td>
<td>+</td>
</tr>
</tbody>
</table>

Unprimed cell culture

| 0 | 3 | - |
| 10 | 1 | - |
| 50 | 15 | - |
| 250 | 200 | - |

* DEAE-dextran was applied to the cells 1 h after they had been primed with interferon and before they were exposed to poly(I).poly(C).
DISCUSSION

Interferon-treated L cells exhibit a specific enhanced susceptibility to the toxicity of dsRNA molecules but not to the toxicity of other agents such as dsDNA, single-stranded RNA, bacterial toxins, snake venoms or metabolic inhibitors (Stewart et al. 1973a). This system may be useful for the detection of dsRNA intermediates in the replicative cycle of RNA and DNA viruses (e.g. vaccinia virus), since vaccinia virus has also been shown to induce a specific lytic effect in interferon-treated L cell cultures, distinguishable from the normal c.p.e. observed in poxvirus-infected cells (Joklik & Merigan, 1966; Horak, Jungwirth & Bodo, 1971; Stewart et al. 1973a). Although the enhanced toxicity of dsRNA in interferon-treated cells has been related to an inhibition of protein synthesis (Kerr, Brown & Ball, 1974), the sequence of events leading to the toxic alteration of the cell and/or inhibition of protein synthesis has not been elucidated. Neither has the relationship between the enhanced toxicity of dsRNA in interferon-treated cells and the well-known sensitivity of interferon-treated cells to the interferon inducing properties of dsRNA.

Various experimental procedures designed to uncouple the cytotoxic and interferon inducing activities of poly(I), poly(C) in interferon-primed L cells failed to do so (cf. Fig. 1, 2, 3b, 4). Alterations of the molecular size of either poly(I) or poly(C), or both, were also unable to dissociate the toxic and interferon inducing properties of poly(I).poly(C) in interferon-treated L cells (Stewart & De Clercq, 1974). These findings would suggest that there is at least one common link in the chain of events leading to toxicity and interferon production in interferon-treated L cells exposed to poly(I).poly(C).

However, the results presented in Tables 1, 2 and 3 and Fig. 3(a) indicate that toxicity and interferon production are not invariably linked. If the mechanisms of toxicity and interferon production are coupled initially, they diverge afterwards. Unlike toxicity, interferon production would require metabolic processes involving transcription and translation, otherwise it would not be inhibited by actinomycin D and cycloheximide (Table 2). On the other hand, the interferon inducing activity of poly(I).poly(C) in L cells could be dissociated from its cytotoxicity provided the cells were 'pulse' treated with either interferon (Fig. 3a) or DEAE-dextran (Table 3). It is conceivable that 'pulse' treatment of the cells with interferon or DEAE-dextran facilitated the interaction of poly(I).poly(C) with the cellular receptor site(s) for interferon induction. This interaction may suffice to transmit the message for interferon induction. However, it may be too weak to lead to a toxic (or at least morphological) alteration of the cell.

To accommodate all results reported herein, the relationship between interferon production and toxicity of poly(I).poly(C) in interferon-primed L 929 cell cultures could be presented as follows:

\[
\text{Poly(I).poly(C)} \quad \downarrow \\
\text{Interaction with cell (membrane) receptor site(s)} \\
\text{Direct alteration of cell membrane} \quad \text{Inactivation of interferon repressor} \\
\text{TOXICITY} \\
\text{Transcription of interferon gene to interferon mRNA} \\
\text{Translation of interferon mRNA to interferon} \\
\text{INTERFERON PRODUCTION}
\]
According to this working model, the common link in the chain of events leading to toxicity and interferon production in interferon-treated L cells exposed to poly(I).poly(C) would be the interaction of poly(I).poly(C) with the cell receptor site(s), presumably situated at the outer surface of the cell (De Clercq & De Somer, 1972b, 1974; Taylor-Papadimitriou & Kallos, 1973). Toxicity would be the direct consequence of an alteration of the cell membrane generated upon interaction of poly(I).poly(C) with the cell. Interferon production, however, would require a few additional steps involving derepression, transcription and translation, since metabolic inhibitors block interferon production but do not affect toxicity. Finally, judicious choice of the assay conditions, e.g. short-term (‘pulse’) treatment of the cells with interferon, may uncouple the interferon production pathway from the toxicity pathway.

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Poly(I).poly(C): interferon and toxicity
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